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Purification and Characterization of *Bacillus subtilis* Protoporphyrinogen Oxidase and Pre-equilibrium Behavior During Oxidation of Protoporphyrinogen IX

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Previous studies indicate that *B. subtilis* protoporphyrinogen oxidase is poorly inhibited by diphenyl ether herbicides. To better understand the basis of this insensitivity, the enzyme was overexpressed as a soluble protein in *E. coli*, purified and characterized. The mechanism of oxidation of *B. subtilis* protoporphyrinogen IX was studied and the enzyme kinetic parameters were determined for protoporpyrinogen IX; K_m and k_{cat} were 6.3 μM and 0.028 h^{-1}, respectively. The enzyme required flavin adenine dinucleotide as a cofactor and its activity was enhanced by 1 mM *n*-octylglucopyranoside. The nonenzymatic oxidation rate was dependent on the concentration of protoporphyrinogen IX, suggesting that the reaction involves a pre-equilibrium step followed by a rate-limiting step.

Keywords: Protoporphyrinogen, Protoporphyrin, Pre-equilibrium, Herbicide, Oxidase.

Introduction

The oxidation of protoporphyrinogen IX (Protogen IX) is the last common step in the biosynthesis of heme and chlorophyll (Beale and Weinstein, 1990). Protoporphyrinogen oxidase (Protox, EC 1.3.3.4) catalyzes the six-electron oxidation of Protogen IX *in vivo* and produces protoporphyrin IX (Proto IX). However, the oxidation of Protogen IX can also occur nonenzymatically (Han *et al*, 1995; Han and Kim, 1996). In this reaction, molecular oxygen is the electron acceptor which is reduced to hydrogen peroxide, as shown in Fig. 5 (Jacobs and Jacobs, 1982).

In plants, Protox is the primary target of a variety of photodynamic diphenyl ether herbicides, such as oxyfluorfen and acifluorefen (Duke and Rebeiz, 1994). The biochemical basis for the herbicidal action of these compounds is competitive inhibition of Protox, which is located in the plastid envelope. When Protox is inhibited, Protogen IX accumulates in the plastid envelope, diffuses into the plasma membrane, and is oxidized by a herbicide-insensitive Protox and/or autooxidation in the plasma membrane in order to produce Proto IX and hydrogen peroxide. Accumulation of Proto IX in the plasma membrane causes lipid peroxidation leading to cellular death in the presence of molecular oxygen and light. All eukaryotic Protox enzymes characterized to date are severely inhibited by diphenyl ether compounds (Lee *et al.*, 1993; Lermontoba *et al.*, 1997). In contrast, *B. subtilis* Protox is poorly inhibited by diphenyl ether herbicides (Jacobs *et al.*, 1990; Dailey *et al*, 1994). Further, it was recently shown that transgenic plants expressing *B. subtilis* Protox are resistant to diphenyl ether herbicides (Choi *et al.*, 1998; Kim *et al.*, 2000; Lee *et al.*, 2000). Due to this important finding, this enzyme has been further studied. *B. subtilis* Protox was cloned and expressed in *E. coli* (Dailey, *et al.*, 1994). In initial efforts to express this enzyme using pBTac and pT7-5 based expression systems, *B. subtilis* Protox was made insoluble and extensive denaturation was needed to purify the recombinant protein (Corrigall *et al.*, 1998). In this study, *B. subtilis* Protox was expressed as a soluble protein in *E. coli* BL21(DE3)PlysS using the pRSETB vector. The recombinant enzyme was purified and the mechanism of its oxidation of Protogen IX was characterized.

Materials and Methods

Chemicals and reagents Proto IX and *n*-octylglucopyranoside were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA). The DNA QIAEX purification kit was purchased from QIAGEN (Hilden, Germany). Restriction enzymes and T4 DNA ligase were obtained from Promega (Madison, Wisconsin, USA). The nickel affinity column was purchased from Invitrogen (NV Leek, The Netherlands). All other chemicals were of the highest grade available.

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Heterologous expression of *B. subtilis* Protox in *E. coli* A plasmid carrying the *B. subtilis* Protox gene cloned into a pBTac I vector (pBTac(protox)) was obtained from H. A. Dailey (University of Georgia, USA). The pBTac(protox) plasmid was digested with *BamHI* and *HindIII*, the 1.4 kb insert was purified with a QIAEX purification kit, and ligated into the protein expression vector pRSETB linearized with *BglII* and *HindIII* in order to produce pRSETB(protox). To express recombinant protein, the pRSETB(protox) plasmid was transformed into the BL21(DE3)pLysS strain of *E. coli.* Transformed cells carrying pRSETB(protox) were inoculated into 0.5 liter of LB with 100 μg/mL ampicillin and 35 μg/mL chloramphenicol and grown at 25°C with 200 rpm shaking until an A_{600} of 0.5 was reached (Kim *et al.*, 1999). The expression of the recombinant Protox enzyme was induced by adding IPTG (1 mM). Cells were grown for an additional 5 h at 25°C and harvested. The induced recombinant Protox protein was found in inclusion bodies if cells were grown for longer than 5 h, or at a temperature higher than 25°C.

Purification of the recombinant Protox enzyme Cells were harvested by centrifugation, resuspended in a 50 mM sodium phosphate buffer, pH 7.4, containing 0.3 M NaCl, 0.5 mM PMSF, 0.2% *n*-octylglucopyranoside, 1% 1,10-phenanthroline and disrupted by sonication. Cellular debris was removed by centrifugation at 100,000 × g, the supernatant was collected, and the crude extract was subjected to Ni-affinity chromatography. The Ni-column was equilibrated with 10 mM HEPES, pH 7.9, containing 5 mM $MgCl_2$, 0.1 mM EDTA, 50 mM NaCl, and 17% glycerol. The crude extract was loaded on the Ni-column, washed and eluted with an equilibration buffer containing gradient of 50 mM-700 mM imidazole. The Protox enzyme was eluted at 500 mM imidazole.

Oxidation of Protogen IX Protogen IX was prepared *via* reduction of Proto IX with a 3% sodium amalgam as previously reported (Dailey and Dailey, 1996). The Protogen IX solution was adjusted to pH 8.0 by dropwise addition of 40% phosphoric acid and stored in liquid nitrogen. Protox activity was assayed by measuring the rate of the appearance of Proto IX, as detected by the fluorescence emission at 622 nm with excitation at 395 nm at 25°C, or by measuring absorbance at 406 nm (ε_{406} = 5,665 M^{-1} cm^{-1}). The assay buffer contained 100 mM sodium phosphate, pH 7.4, 0.1 mM EDTA and 0.1% Tween 20. For reconstitution with flavin nucleotides, the reconstitution mixture contained 380 nM Protox, 5 μM flavin adenine dinucleotide (FAD) or flavin adenine mononucleotide (FMN) in the assay buffer (Kil *et al.*, 1998).

Results and Discussion

Purification and characterization of *B. subtilis* Protox *B. subtilis* Protox was expressed in *E. coli* in a soluble form and purified by affinity chromatography using N-terminal His-tag. Nickel affinity chromatography provided a one-step purification and yielded homogeneous recombinant Protox (Fig. 1). Kinetic parameters that were determined for Protogen IX; K_m and k_{cat} were 6.3 μM and 0.028 h^{-1}, respectively.

B. subtilis Protox was also reconstituted with FAD or FMN

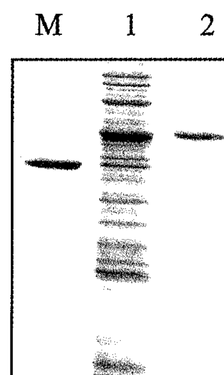

Fig. 1. SDS-PAGE of Protox overexpressed in *E. coli* BL21(DE3)pLysS. After a 5 h induction with 1 mM IPTG, cells harboring the plasmid pRSETB(Protox) were harvested, lysed, and the Protox was purified as described in Materials and Methods. Samples were electrophoresed on a 12% polyacrylamide gel. Lane 1: molecular weight marker (6-deoxyerythronolide B hydroxylase, 45 kDa), lane 2: crude extract, and lane 3: purified Protox.

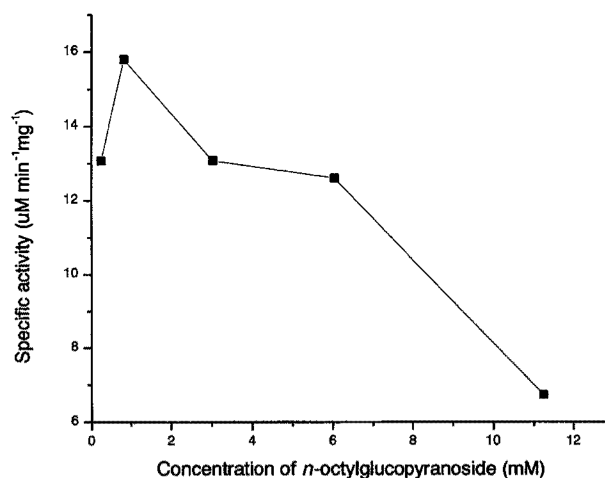

Fig. 2. Effect of n-octylglucopyranoside on the activity of Protox. *n*-Octylglucopyranoside was added to the assay buffer containing 100 mM sodium phosphate, pH 7.4, 0.1 mM EDTA and 0.1% Tween 20 and the specific activity was measured as described in the Materials and Methods.

and the activity was measured. The assay mixture with 5 mM FAD had a 2- to 3-fold higher activity in the presence of this cofactor; in contrast, conditions without FAD or with FMN are not optimal for Protox (data not shown). This result indicates that FAD is the required cofactor for *B. subtilis* Protox. This result is consistent with previous analysis of the amino acid sequence of *B. subtilis* Protox by Dailey *et al.* (1994), who proposed a putative consensus sequence for dinucleotide binding in this enzyme.

Effect of *n*-octylglucopyranoside on Protox activity The autooxidation of Protogen IX depends on the ionic strength of the solvent and the presence of lipids (i.e., palmitic acid) as

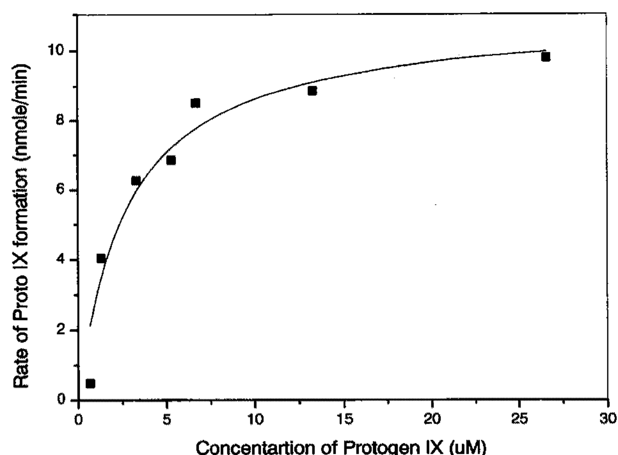

Fig. 3. Pre-equilibrium behavior of the oxidation of Protogen IX. The Protogen IX oxidation rate was determined by measuring the rate of appearance of Proto IX as detected by fluorescence emission at 622 nm with excitation at 395 nm at 25°C. The assay buffer contained 100 mM sodium phosphate, pH 7.4, 0.1 mM EDTA, and 0.1% Tween 20.

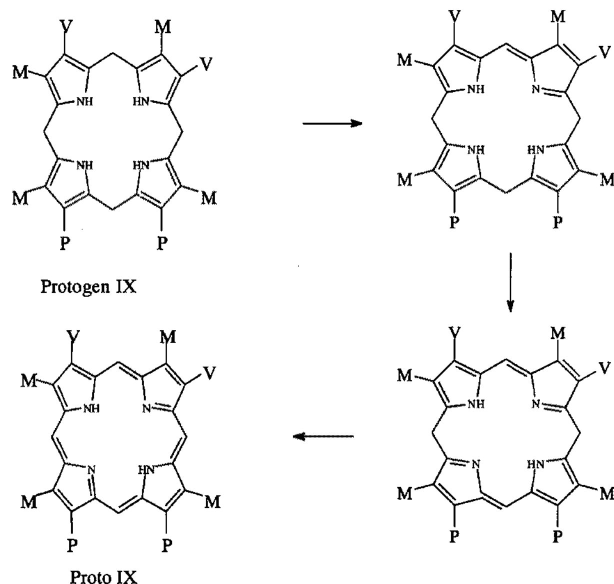

Fig. 4. Hypothetical intermediates in the mechanism of the oxidation of Protogen IX. The two-electron oxidation of Protogen IX produces the first intermediate and the subsequent two-electron oxidation produces the second intermediate, and the final two-electron oxidation yields Proto IX. M = $-CH_3$, V = $-CHCH_2$, P = $-CH_2CH_2COOH$.

well as the reducing agents such as DTT (Han *et al.*, 1996). This result suggests that the autooxidation of Protogen IX might be enhanced by a hydrophobic environment in the absence of DTT. Thus, the Protox reaction rate was measured in the presence of a variable concentration of *n*-octylglucopyranoside. As shown in Fig. 2, the specific activity of Protox was maximal at 1 mM *n*-octylglucopyranoside. Because Proto IX is more hydrophobic than Protogen IX (Lee *et al.*, 1993), this result may be explained by the hypothesis that a hydrophobic environment stabilizes the product-like hydrophobic transition state in the Protox-catalyzed oxidation of Protogen IX (Han *et al.*, 1996).

Pre-equilibrium kinetic behavior during oxidation of Protogen IX The enzymatic oxidation and nonenzymatic autooxidation of Protogen IX are similarly affected by a hydrophobic environment. Thus, an experiment was carried out to determine if the nonenzymatic oxidation rate of Protogen IX depends on its concentration. As shown in Fig. 3, the nonenzymatic autooxidation rate saturates as the concentration of Protogen IX increases, which is similar to the Michaelis-Menten kinetic behavior of Protox. The saturation kinetics indicate that the oxidation mechanism involves at least one pre-equilibrium step prior to the rate limiting step in the reaction. Since the oxidation of Protogen IX is a six-electron oxidation, it is expected that at least two intermediates are involved in the oxidation mechanism. The first intermediate is likely to be a two-electron oxidized form, and the second intermediate is likely to be a four-electron oxidized form of Protogen IX. Fig. 4 shows a hypothetical reaction mechanism in which there are two intermediates and three steps. The first step is not likely to be a rate-limiting step, because the pre-equilibrium kinetic behavior was observed in the oxidation of Protogen IX. Thus, the second or third steps in Fig. 4 could each be the rate-limiting step. It would be useful to look for a kinetic isotope effect using deuterium-labeled Protogen IX to identify which of these two steps is rate-limiting.

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